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We are attempting to determine the secondary structure of the repetitive eggshell protein from Schistosoma mansoni using synthetic peptides. CD spectroscopy, Fourier transform Infra-red spectroscopy, titration experiments and computer modeling, all suggest that this protein adopts a left-handed alpha-helix in aqueous solution, the first time such a structure has been reported in a natural protein.

Experiments in vivo suggest that pH and calcium play important roles in regulating the polymerization of schistosome eggshell. We have developed a novel method for detecting DOPA proteins. These proteins are only found in the vitellaria of mature female schistosomes.

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PRINCIPAL INVESTIGATOR: John S. Cordingley and C.Russell Middaugh

CONTRACTOR: University of Wyoming

CONTRACT TITLE: Phenol oxidase mediated protein cross-linking.

START DATE: 1 June 1987

RESEARCH OBJECTIVE:

The aim of this research is to investigate the secondary structure of the highly repetitive schistosome eggshell protein known in our laboratory as F4. The schistosome eggshell is cross-linked by "quinone tanning" apparently catalyzed by a copper dependent phenol oxidase and a secondary aim of this project is to characterize the enzyme(s) responsible for this process and to attempt to isolate, clone and sequence the genes for this enzyme(s).

THE STRUCTURE OF MODEL SYNTHETIC PEPTIDES.

One of the primary aims of this project was to model the repetitive eggshell protein known as F4 using synthetic peptides. The basic repeat Gly-Tyr-Asp-Lys-Tyr (GYDKY in single letter code) was synthesized as 1,2,3 and 6 repeats of this basic five amino acid repeat, yielding peptides of 5, 10, 15 and 30 amino acids. These peptides were examined by several physical techniques as solutions in a variety of solvents and as dry layers and powders by FTIR.

CIRCULAR DICHROISM.

The 30 amino acid peptide (F4-6) comprising 6 repeats (GYDKY x6) was examined first since it was expected to show the highest degree of structure (which indeed turned out to be the case). The CD spectrum in water is shown in Figure 1A. The spectrum shows a positive ellipticity around 230nm and a pronounced negative ellipticity at 188. This is quite unlike any of the well characterized spectra one obtains with right-handed alpha-helix, beta sheet or un-ordered structure. This spectrum is characteristic of left handed alpha-helices a structure which was predicted by Branson, Cory and Pauling in the 1950's but has never been observed in any structure determined to this date. This unexpected and unprecedented observation needs some fairly vigorous controls since our first reaction was to suspect some kind of misleading artifact. The following control experiments have however all failed to disprove our initial interpretation.

1) CD spectra determined in Trifluoro-ethanol reveal a typical right handed alpha helix spectrum (Fig. 1B).

2) CD spectra of 1 repeat (5 amino acids) and 2 repeats (10 amino acids) show very few of the features of the 30 amino acid peptide.

3) CD spectra of the 30 amino acid peptide in strongly denaturing conditions show little if any secondary structure (Figure 1C).

4) Increasing temperature or lithium perchlorate concentrations induce denaturation and loss of the major features of the CD spectrum with sharp, apparently cooperative melting transitions, consistent with the loss of a repetitive structural motif such as an alpha-helix (Figure 1B).

FOURIER TRANSFORM INFRA RED SPECTROSCOPY.

FTIR spectra of dry films, D^2O solutions and intact eggshell all give spectra

previously suggested to be typical of putative left handed alpha-helices. These spectra will not be described in detail here but will be presented in full in a forthcoming manuscript. The most significant observation in this series was probably that obtained with an FTIR microscope upon intact eggshells suggesting that the eggshell itself contains significant amounts of left-handed alpha-helix. Due to their insolubility CD spectra are not obtainable from eggshells, therefore the FTIR microscope is the only way we currently have of obtaining information about the eggshells secondary structural features.

COMPUTER MODELING.

We have modeled all the various repetitive potential secondary structures of the F4-6-repeat peptide using Biosym's software packages Discover and Insight. The initial energies of the structures showed that the left hand alpha helix had the lowest energy (wet or dry) and after minimization of the energies of the right and left handed alpha helices (both wet and dry) the left-handed alpha helix possessed by far the lowest energy. Figure 2 is a stereo pair of the resulting structure after minimization in the presence of water.

TITRATION.

The predicted structure showed several features that suggested further experimental tests of the predicted structure. Most significantly the Lys and Asp residues all participate in ion pairs suggesting that the pKa's of both of these residues should be shifted due to charge stabilization, which proved to be the case, Asp being shifted down about 1 pH unit and Lys up by a similar amount. In addition the minimized structure of the left-handed helix shows that one of the two tyrosines in each repeat is hydrogen bonded to the Asp carboxyl group and should have its pKa shifted in consequence. Optical titration of the tyrosines shows that indeed the tyrosines show two distinct pKa's consistent with the predicted two environments of the tyrosine hydroxyls.

1-D AND 2-D NMR.

Dr. Middaugh has recently moved to Merck Sharpe and Dohme and has been able to obtain NMR data on the F4-6 peptide both in 1-D and 2-D modes. Currently we are attempting to assign some of the residues unambiguously but the repetitive nature of the peptide renders this tricky. Thus currently the NMR data neither confirms or denies the left handed alpha helical structure. The one dimensional NMR spectrum strongly suggests that the tyrosine hydroxyls are in two distinct environments. We hope to resolve this in the near future.

DOPA PROTEINS IN FEMALE Schistosoma mansoni.

Herb Waite's pioneering work has shown that a special group of proteins with structural roles such as the mussel glue proteins and Fasciola eggshell precursor proteins have their tyrosine residues oxidized to DOPA prior to secretion. We have been trying to determine whether schistosomes have processed their eggshell proteins in a similar fashion. We have been unable to demonstrate DOPA proteins convincingly or reproducibly with "Arnow's Stain" although Fasciola DOPA protein kindly supplied by Herb Waite stains reproducibly in our hands.

NOVEL SILVER STAINING OF DOPA-PROTEINS.

However we have discovered that we can detect DOPA by its ability to reduce silver in alkaline conditions, making the detection of DOPA proteins by silver staining possible without staining any other kinds of protein at all. A further refinement is to silver stain gels with and without oxidation. DOPA proteins stain much more rapidly and much more intensely before oxidation. Indeed their is a point during the development of such unoxidized gels when only the DOPA-proteins are stained.

This technique can be used histologically, enabling us to demonstrate that the

vitelline cells, where the eggshell proteins are synthesized, are the sole sites of silver deposition in an acid-ethanol fixed female worm. All of these proteins and staining effects are only seen in mature females, males and immature females do not stain in this way or show these putative DOPA proteins.

STUDIES ON THE PHENOL OXIDASE(S).

We are trying to elucidate the role of phenol oxidase enzymes in the processes we are characterizing. There may be one or two enzymes involved and our experiments to date have not enabled us to purify the enzyme(s) involved, largely due to their insolubility. This work continues.

INTRACELLULAR LOCALIZATION.

Using tyrosine methyl ester we have been visualizing the in vivo location of phenol oxidase by the fluorescent product formed. This enzyme is inhibited by diethyl dithiocarbamate suggesting it is copper dependent, however the realization that the majority of the tyrosines in the eggshell proteins are converted to dihydroxyphenylalanine (DOPA) intracellularly raises the question of which enzyme we are detecting, or indeed whether one enzyme performs both tasks ie, converting tyrosine to DOPA inside the cells and DOPA to quinone extracellularly during eggshell cross-linking. The widespread, diffuse location of fluorescent phenol phenol oxidase staining suggests to us that we are staining the tyrosine to DOPA activity possibly inside the endoplasmic reticulum?

THE CELLULAR MECHANISM CONTROLLING EGGSHELL POLYMERIZATION.

VESICLE pH.

In common with many regulated secretory vesicles the eggshell protein vesicles appear to be acid compartments. Indeed it appears as if the low pH of these vesicles may be one of the major factors controlling the polymerization reaction. One extraordinary feature of the newly polymerized eggshell is that the reaction products formed are highly autofluorescent. In normal female worms the ONLY fluorescent structure is the newly formed eggshell. Any treatment that induces polymerization induces this intense fluorescence. Treatment of living worms with ammonium chloride, a treatment known to elevate the pH of intracellular acid compartments, induces this fluorescence throughout the vitellaria of the worm.

THE ROLE OF CALCIUM.

Extending the analogy with other systems which produce acidic, regulated secretory vesicles (such as the adrenal medulla), we guessed that the exocytosis of these vesicles may be controlled by calcium. This was tested by the use of calcium plus calcium ionophore which strongly induced fluorescence in a distribution apparently identical to that induced by ammonium chloride.

SUBCELLULAR STRUCTURE AND DYNAMICS.

Thus we now can summarize the processes that we observe at both the cellular and the molecular level. The eggshell proteins are synthesized and secreted into the lumen of the ER. They are then processed to convert the tyrosine side chains to DOPA, generating what Herbert Waite has termed "presclerotized proteins" These proteins presumably pass through the golgi and are packaged into vesicles which are acidified at this point and remain so until secretion and polymerization. These small "post-golgi" vesicles fuse with other vesicles to form the large characteristic "vitelline droplets" observed in mature vitelline cells.

The main feature of these vesicles is that they contain multiple electron dense droplets of eggshell precursor material. Thus these vesicles contain a stable emulsion of protein droplets. This emulsion (we hypothesize) is stabilized by the low pH of these vesicles. These cells pass down the vitelline duct and are "injected" in batches into the

ootype (a process which we have observed in worms in culture) at the start of each individual eggshell production cycle (which lasts about 5 minutes). At this point some stimulus (a hormone?) is produced by the ootype which triggers a calcium pulse into these cells which then exocytose their eggshell precursor material. Our observations of eggshell formation in cultured female schistosomes showed that it involves a vigorous agitation of the contents of the ootype at this time accompanied by the cross-linking reaction which generates the vivid fluorescence of the eggshell. In less than 5 minutes the eggshell is hardened sufficiently to allow the worm to pass the egg into the uterus prior to deposition. The cycle is then repeated.

PROJECTED STUDIES FOR YEAR THREE.

The studies of the synthetic peptides will be extended to include other regions of the F4 protein, the complete sequence of which will shortly be finished from a genomic clone of the single gene in our schistosome strain. The sequence so far completed shows that the repeats continue for the majority of the length of the protein but towards the amino terminus a slightly more complex pattern of repeats is established. The repeat (GYDKY-GSDKY-GYEK) repeats exactly many times. We have initiated computer modeling studies and synthetic peptide analogues of this structure have been ordered.

The Histidine rich carboxy terminus of the F4 protein has also been made as a synthetic peptide and structural studies of this peptide will be completed, along with the parallel computer modeling and energy minimizations. These computer calculations are running as I write. However our experience suggests that these calculations will take many weeks. The F4-6 peptide (wet structure) took 55 days constant computing to come up with the structure in figure 2. Fortunately we have our own VAX. Unfortunately it is not a CRAY.

The studies of the phenol oxidase will be continued and the in vivo control of eggshell polymerization studies pursued to try to improve our understanding of the control of the eggshell cross-linking reactions. We believe that these worms can teach us a great deal more about the miniature chemical engineering that they perform during eggshell polymerization. Several features of the events we are observing suggest that we do not fully understand the processes occurring.

PUBLICATIONS AND REPORTS.

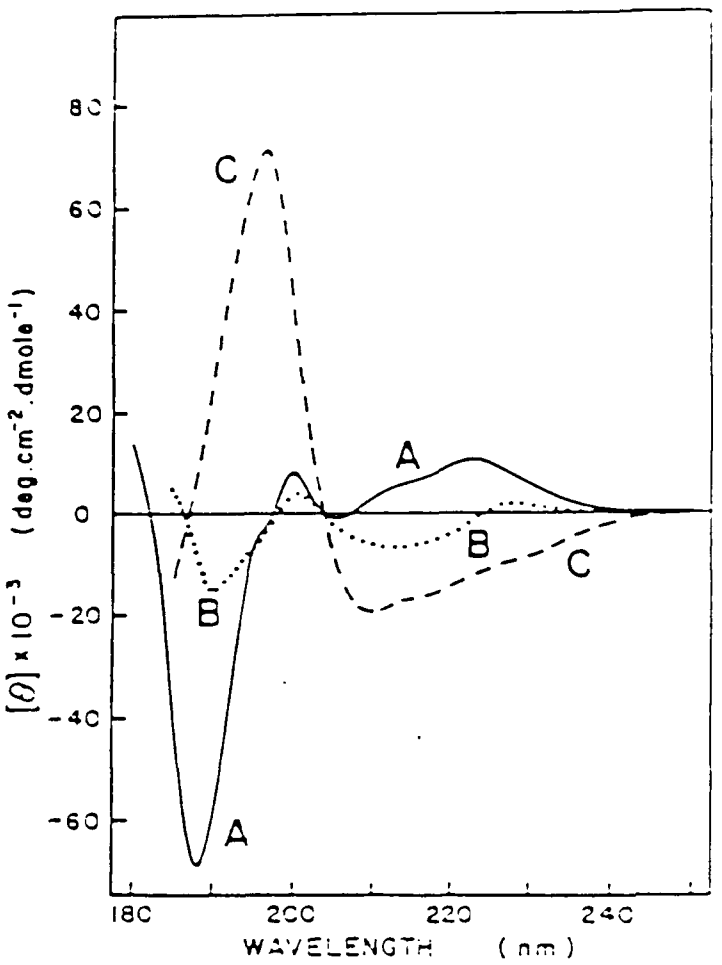
A paper has been submitted to Science (a copy is attached). Discussion with the editors resulted in the paper being returned pending NMR data to confirm our radical claims. The editors then want to see the paper again. Therefore we are holding up submission with the intention of submitting to Science when we have confirmatory evidence.

Two other manuscripts are in preparation at the time of writing of this report. Tentative titles are:

"Detection of DOPA proteins by a novel silver staining procedure."

and "Eggshell polymerization is regulated by pH and calcium in Schistosoma mansoni."

Seminars have been presented about this work at the University of Colorado in Boulder, and at the National Jewish Hospital Research Center in Denver.



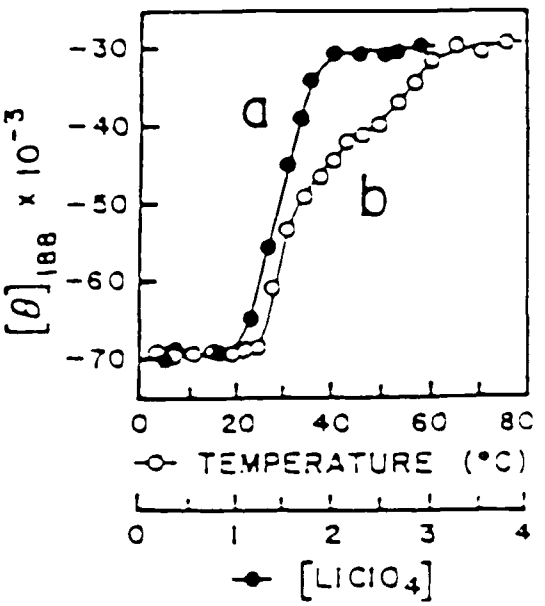


Figure 1A: CD spectra were recorded at 20°C with a JASCO J500A spectropolarimeter at a peptide concentration of 0.1 mg/ml employing 1 mm cells. Protein concentrations were determined by Tyrosine absorbance. CD spectra of (A) F4-6 in 20 mM sodium phosphate, pH 6.5. (B) F4-6 in 20 mM sodium phosphate, 4 M $LiClO_4$, pH 6.5 heated to 90°C for 30 minutes prior to analysis, (C) F4-6 in trifluoroethanol.
Figure 1B This shows the (a) effect of $LiClO_4$ concentration on the CD spectrum of F4-6 at 188 nm and 20°C and (b) the effect of temperature on the 188 nm negative ellipticity minimum.

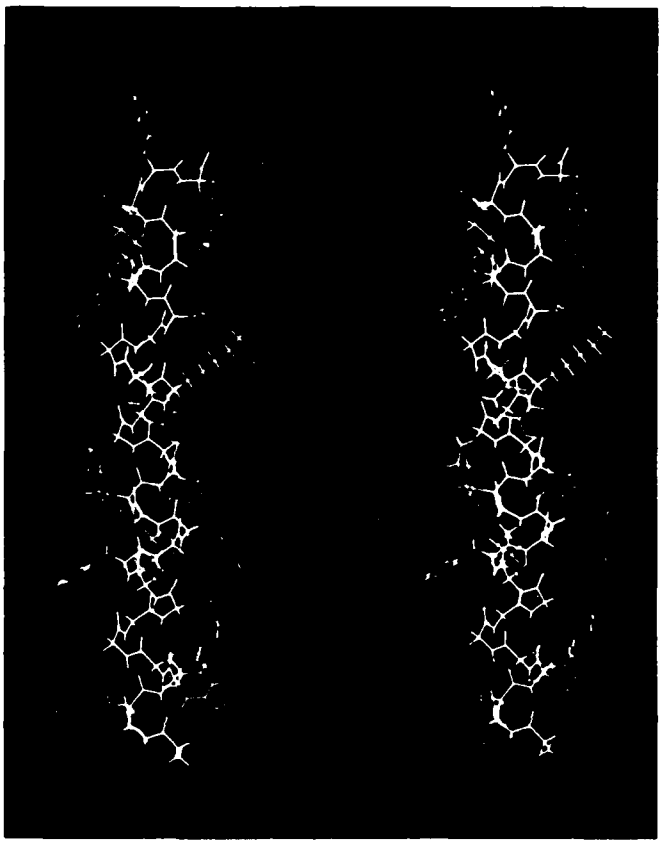


Figure 2.

Stereo graphics display of the F4-6 peptide in the left handed alpha-helical configuration, following energy minimization with a 3 Angstrom hydration layer. The energy of the hydrated peptide was minimized by the conjugate gradients method to a maximum derivative of 0.01 kcal/Angstrom, using DISCOVER (Biosym Inc.). The photocopy preserves the stereo image and structural details accurately but the original colors are unfortunately lost. Picture generated by INSIGHT (Biosym Inc.) using an Evans and Sutherland PS300 graphics system.